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16. ABSTRACT

The use of chlorine as a drinking water disinfectant is known to produce a variety of chlorinated and oxidized derivatives of organic matter present in the source water. Humic substances, which represent the major fraction of the organic matter in natural waters, have been shown to give rise to numerous carcinogenic and mutagenic substances upon chlorination. Attempts to fully characterize and/or identify the mutagenic reaction products were made using various isolation/concentration techniques in combination with GC/MS. Halogenated aldehydes, acids, ketones, nitriles, alkenes, and aromatic compounds were identified. The similarity of mutagenic compounds identified in chlorinated drinking water and in chlorinated solutions of commercial humic acid suggests that the reaction of chlorine with natural aquatic humic material is a likely source of mutagen formation in drinking water. While much of the mutagenic activity could be extracted, attempts to recover a significant portion of the activity from gas chromatographic columns were unsuccessful. Additional studies were conducted to determine the potential for the in vivo formation of halogenated or oxidized derivatives of endogenous organic material resulting from the ingestion of residual chlorine in potable water. GC/MS analyses of extracts of stomach contents and plasma, taken from fasted and non-fasted Sprague-Dawley rats dosed with NaOCL by gavage, demonstrated the in vivo formation of di- and trichloroacetic acids. Chloroform was identified in the stomach contents of the fasted and non-fasted animals dosed with NaOCL but was not detected in the control groups.

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REACTIONS OF CHLORINE IN DRINKING WATER, WITH HUMIC ACIDS AND *IN VIVO*

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The use of chlorine as a drinking water disinfectant is known to produce a variety of chlorinated and oxidized derivatives of organic matter present in the source water. Humic substances, which represent the major fraction of the organic matter in natural waters, have been shown to give rise to numerous carcinogenic and mutagenic substances upon chlorination. Attempts to fully characterize and/or identify the mutagenic reaction products were made using various isolation/concentration techniques in combination with GC/MS. Halogenated aldehydes, acids, ketones, nitriles, alkenes, and aromatic compounds were identified. The similarity of mutagenic compounds identified in chlorinated drinking water and in chlorinated solutions of commercial humic acid suggests that the reaction of chlorine with natural aquatic humic material is a likely source of mutagen formation in drinking water. While much of the mutagenic activity could be extracted, attempts to recover a significant portion of the activity from gas chromatographic columns were unsuccessful.

Additional studies were conducted to determine the potential for the *in vivo* formation of halogenated or oxidized derivatives of endogenous organic material resulting from the ingestion of residual chlorine in potable water. GC/MS analyses of extracts of stomach contents and plasma, taken from fasted and non-fasted Sprague-Dawley rats dosed with NaOCL by gavage, demonstrated the *in vivo* formation of di- and trichloroacetic acids. Chloroform was identified in the stomach contents of the fasted and nonfasted animals dosed with NaOCL but was not detected in the control groups.

INTRODUCTION

The disinfection of drinking water with chlorine is known to produce a variety of chlorinated derivatives by reaction of the chlorine with the organic substances found in the source water.[1-4] Substantial evidence has been accumulated that humic substances, which constitute approximately 30-50 percent of the dissolved organic carbon in water[5], are the principal precursors of trihalomethanes (THMs) and numerous other chlorinated organic species that are present in drinking water as a result of water chlorination.[6-9] Recent studies from this laboratory have demonstrated that the chlorination of both fulvic and humic acids results in the formation of direct acting mutagenic chemicals.[10-11] The similarity of mutagenic compounds identified in chlorinated drinking water and in these chlorinated solutions of humic acid suggests that the reaction of chlorine with natural aquatic humic material is a likely source of mutagen formation in drinking water.[9] The present report describes our work to date on the characterization and identification of reaction products resulting from the chlorination of humic material.

The potential for the <u>in vivo</u> production of mutagenic products formed by the reaction of ingested residual chlorine with endogenous organic material is another area of major concern related to drinking water disinfection. The fact that chlorine is known to react with amino acids to form dihaloacetonitriles[12], that exhibit mutagenic activity in the Ames Salmonella bioassay and tumor initiating activity in the Sencar mouse skin bioassay[13], supports this concern.

EXPERIMENTAL

Preparation of Chlorinated Humic Material

Humic material was purchased from Fluka AG (Switzerland). The preparation and chlorination of the humic material was performed according to Meier et al.[11] at a concentration of 1 g/L total organic carbon (TOC), using a 1:1 ratio of chlorine equivalents per mole of carbon. The pH 7 soluble portion of the Fluka humic material before chlorination was found to contain a humic acid:fulvic acid ratio of approximately 10:1, using pH selective precipitation.[14]

Molecular Weight Fractionation

Chlorinated solutions of the humic material were fractionated by diafiltration using an Amicon stirrer/ultrafiltration cell (Model 52) equipped with Nucleopore® ultrafiltration membranes having nominal molecular weight cut-offs of 500; 5,000; and 50,000.

A 30-ml aliquot of the chlorinated humic solution was placed in the filtration cell containing a 500 M.W. cutoff filter and a pressure of 70 psi was applied using helium gas. The cell volume was held constant by the continual addition of sterile distilled water from the reservoir at a rate equal to the diafiltration output. Following collection of 210 mL of filtrate (M.W. <500), the retentate (M.W. >500) was transferred to another unit and the process repeated for the 5,000 and 50,000 M.W. cut-off filters.

Liquid-Liquid Extraction

Aliquots of chlorinated humic material at pH 2.5 were extracted with two portions of diethyl ether at a solvent:sample ratio of 1:2. Following centrifugation to separate the layers, the ether extracts were combined, dried with sodium sulfate and concentrated 200X by Kuderna-Danish evaporation. Portions of the aqueous layer and concentrated ether extract were saved for mutagenesis testing along with the original samples. GC/MS analyses and quantification were also performed on a separate portion of the ether concentrate containing internal standards.[9]

Closed-Loop stripping (CLS) Analysis

Closed-loop-stripping analyses were performed on the aqueous chlorinated humic acid solution to provide qualitative information on compounds that were present below the detection limits of the liquid-liquid extraction procedure. The CLS system and conditions used for the analyses were reported by Coleman et al.[15]

Steam Distillation

Three-hundred mL of an aqueous solution of the chlorinated Fluka humic material was placed in a distillation apparatus[16] equipped with a special condensate collection chamber designed to concentrate volatile, polar, low molecular weight organics. The flask temperature was controlled to provide a distillate at a rate of approximately 1 mL/90 min.

GC/MS Analyses

GC/MS analyses were performed using a Carlo-Erba Fractovap 4160 gas chromatograph and a Finnigan 3300 quadrupole mass spectrometer equipped with an Incos 2300 data system. The gas chromatograph utilized a 60 m x 0.25 mm i.d. wall-coated-open-tubular (WCOT) fused silica SE-30 column for separation of compounds in the steam distillate and CLS samples. The column vent (20:1 ratio) was opened 30 sec after injection. The helium carrier gas flow rate was approximately 3 mL/min at 25°C at a linear velocity of 25 cm/sec. The injector temperature was maintained at 260°C. Samples were injected at an oven temperature of 20°C, held for 6 min, then programmed to 250°C at a rate of 4°C/min. Mass spectra were acquired at the rate of 1 spectrum/2 sec over a mass range of 14-450 amu at 70eV. A 30 m x 0.25 mm i.d. DB 1701 WCOT fused silica capillary column was used for the GC analysis of liquid-liquid extracts. The oven temperature was programmed at a rate of 6°/min and mass spectra were acquired at a rate of 1 spectrum/sec. All other GC conditions were as listed above.

Mutagenicity Testing

Solutions of chlorinated humic substances or organic solvent extracts were tested for mutagenicity using the Salmonella histidine reverse mutation assay described by Ames et al.[17] The assays were performed with S. typhimurium his$^-$ strains TA98 and TA100. Tests were made with and without rat liver homogenate fraction (S9).

In Vivo Studies

GC/MS analyses were performed on the plasma and stomach contents of rats dosed orally with a single administration of 7 mL of an 8 mg/mL solution of NaOCl at pH 7.9. The methods for sample preparation and analyses have been described by Mink et al.[18] Attempts at compound identification were also performed on urine samples collected from rats dosed with daily administrations of 8 mg and 16 mg (NaOCl)/kg/day for eight days. Following the final dose the rats were placed in metabolism cages and the urine collected in water cooled vials. The urine samples from each dose group (10 animals/group) were pooled. The extraction and concentration of the urine samples were performed using the procedure of Yamasaki and Ames.[19] The acetone eluates were concentrated and subjected to GC/MS analyses.

RESULTS AND DISCUSSION

Since it has been demonstrated previously in this laboratory[10,11] and by Kringstad et al.[20] that the chlorination products of humic materials exhibit mutagenic activity in the Ames test, various fractionation and isolation procedures were utilized in an attempt to characterize/identify the mutagenic compounds. The molecular weight fractionation of chlorinated humic material by diafiltration suggested that the majority of the mutagenic activity was in the <500 M.W. fraction (Table I). The finding of a significant portion of the mutagenic activity in the greater than 50,000 M.W. fraction for TA98, however, was unexpected. An explanation may be the binding or asso-

ciation of mutagens possessing hydrophobic properties to dissolved humic material, similar to that discussed by Carter and Suffet[21] for the pesticide DDT. The inability to account for a higher percentage of the mutagenic activity in the whole sample following fractionation was probably attributable to adsorption losses on the ultrafiltration membranes and to the detection limits of the assay.

Utilizing the liquid-liquid extraction procedure, diethyl ether recovered nearly all the mutagenic activity present in the chlorinated solution of humic material.[9] The ability of diethyl ether to recover approximately 100 percent of the activity, as compared to only 40 percent with methylene chloride, strongly suggests the presence of oxygen containing functional groups in the mutagenic compounds. GC/MS analyses of the same methylene chloride and ether extracts (but without solvent exchange), which were tested for mutagenicity, revealed the presence of a number of halogenated organic compounds with carbonyl functional groups (Table II). Confirmed compound identifications were made for chloroform; dichloroacetonitrile; trichloroacetonitrile; dichloroacetic acid; trichloroacetic acid; 1,1-dichloropropanone and 1,1,1-trichloropropanone. Confirmations were based on mass spectra and relative retention times of authentic standards run under identical GC/MS conditions as the sample. Tentative identifications were made for the other compounds given in Table II for which authentic standards were not available. These were based on a manual interpretation of the data. Table II also indicates the compounds that have been previously identified in

drinking water. The similarity of identified substances, a number with known mutagenic activity, suggests that the reaction of chlorine with humic material is a likely source of mutagen formation in drinking water.

Two other isolation techniques were used in an attempt to identify chlorinated reaction products that might be present. Closed-loop-stripping (CLS) was selected because it is an ultrasensitive method for detecting purgeable organics. This technique resulted in the tentative identification of several non-polar chlorinated reaction products (Table II-Miscellaneous). Use of a distillation technique was made to determine if low molecular weight, moderately-polar, compounds that azeotrope with water were responsible for the mutagenic activity. Some success was attained in that additional compounds, including several known mutagens (primarily aldehydes) were identified in the condensate (Table II); which had not been previously observed in the CLS or liquid-liquid extracts. However, the recovery of mutagenic activity in the distillate following fractionation by steam distillation was less than 15% of the total sample activity. Synergistic or antagonistic interactions of the components did not appear to be a factor, since the recombined pot and distillate fractions gave an additive mutagenic response (Table III). The loss of mutagenic activity is difficult to explain since autoclaving of similar samples in this laboratory resulted in minimal losses of activity.

An attempt was made to estimate the contribution of the mutagenic

activity of the identified known mutagens for which quantitations were made[9], to the overall mutagenicity level of the humic acid sample which had been chlorinated at neutral pH. This estimation was done by multiplying the specific mutagenicities (net revertants/mg) of the compounds by their concentration in the original sample, and then dividing by the mutagenicity level (net revertants/ml) of the sample. Based on this calculation, the identified mutagens in Table 2 only accounted for 5-10% of the total mutagenicity present.

Because of the lack of standards for the compounds tentatively identified in Table II, quantification as well as the determination of the specific mutagenicity on a compound by compound basis was not possible. Consequently, the importance of these compounds in terms of their contribution to the overall mutagenicity of the samples is not known. It is interesting to note the identification of several chlorinated propenals (i.e., chloroacroleins), since these compounds have been reported by Rosen et al.[22] to be very potent mutagens. The presence of 2-chloropropenal following humic acid chlorination, has also been reported by Kringstad et al.[20]

An attempt was made however, to determine whether the compounds responsible for the mutagenic activity were amenable to the GC/MS procedure described. Preliminary heat stability tests were performed on the residue of ether extracts of chlorinated humic acid solutions. Ames testing indicated that approximately 50% of the mutagenic activity was associated with compounds that were degraded when heated to 220° in an evacuated flask for 1 minute (Figure 1). The mutagenic

activity also appeared to be associated with relatively nonvolatile compound(s), based on recovery values of about 90% of the mutagenic activity following lyophilization of chlorinated solutions of humic material, as previously reported.[11]

Subsequent trapping studies of the gas stream exiting the GC system, using ether traps agreed with the preliminary findings, as less than 10% of the mutagenic activity was recovered (Figure 2). An on-column injection technique which avoided the high injector block temperature was also tried with essentially the same results. These findings strongly suggest that the majority of the mutagenic activity is not associated with compounds quantified in Table II.

Because of the similarity of mutagenic compounds common to solutions of lignin and humic acid, the separation procedure for strong acids recently reported by Holmbom et al.[23] for the fractionation of chlorinated Kraft pulp was tried. Approximately 70% of the mutagenic activity was retained in the strong acid fraction supporting the possibility that a chlorinated hydroxy furanone such as the 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)furanone, tentatively identified by Holmbom et al.[23], may be responsible for the activity in chlorinated humic acid solutions, as well as chlorinated pulp effluents. Also supporting this possibility is that prior work in this laboratory[11] demonstrated the loss of mutagenic activity with increases in pH and temperature similar to that shown for the chlorinated hydroxy furanone. Attempts to analyze mucochloric acid, a closely related compound, by direct GC/MS resulted in a nonquantitative decarboxylation

of the acid to 2,3-dichloropropenal. Similarly, decarboxylation of the proposed highly mutagenic chlorinated hydroxy furanone should produce a trichloroisobutenal. Dichlorobutenals have been tentatively identified by GC/MS.

The essential issue of the single dose rat study was to establish the presence of chlorinated reaction products in vivo. GC/MS analyses by Mink et al.[18] in this laboratory demonstrated the in vivo formation of dichloroacetonitrile, dichloroacetic acid (DCA), trichloroacetic acid (TCA) and chloroform. Chloroform was detected in the stomach contents, while DCA and TCA were detected in both the stomach contents and plasma of the fasted and non-fasted dosed animals. Dichloroacetonitrile, a known chlorination by-product of several amino acids[12], was found only in the stomach contents of non-fasted rats.

The multiple dose study utilized a sodium hypochlorite concentration that was more consistent with drinking water intake and that had produced cardiovascular effects in pigeons and rabbits[24] and spermhead abnormalities in the mouse[25]. GC/MS analyses of the urine extracts failed to demonstrate the presence of any chlorinated reaction products.

SUMMARY

The by-products of the chlorination of humic material were similar to those found in drinking water (Table II).

The majority of the mutagenic activity of chlorination by-pro-

ducts was associated with compounds that were heat labile at 220°C, relatively non-volatile (could be lyophilized), and that have a molecular weight of less than 500 daltons.

Only 5-10% of the mutagenic activity can be accounted for by specific compounds identified in Table II.

Less than 10% of mutagenic activity was recovered in diethyl ether traps using the GC procedure described.

Approximately 70% of thee mutagenic activity was associated with compounds that are strongly acidic. This finding coupled with heat lability data, instability at high pH, low molecular weight, the likelihood of carbonyl or hydroxyl functional groups (extraction data), the inability to recover the activity following direct GC injection, and the fact that lignin structural units are present in humic material of terrestial origin suggests that chlorinated hydroxy furanones may also be responsible for an appreciable portion of the activity in chlorinated humic material as well as chlorinated Kraft pulp effluent.

Further research is required to determine whether chlorinated hydroxy furanones are responsible for an appreciable portion of the mutagenic activity. Synthesis of appropriate standards will be required.

Future <u>in vivo</u> studies require the development of techniques with increased sensitivity to detect µg and ng quantities of chlorinated reaction products.

DISCLAIMER

The research described in this paper has been peer and administratively reviewed by the U.S. Environmental Protection Agency and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Table I. Molecular Weight Distribution of Chlorinated Humic Material After Diafiltration

Fraction	TOX (Mg/L)	TOX % Total	TA98 Mutagenesis[a] Net Revertants/mL	TA98 % Total	TA100 Mutagenesis[a] Net Revertants/mL	TA100 % Total
Whole Sample	425	100	242 ± 17	100	2,467 ± 48	100
<500	200	47	86 ± 12	36	1,465 ± 33	59
>500	138	33	44 ± 17	18	-	(<18)
>500 <5,000	61	14	-	(<17)	-	(<18)
>5,000	59	14	-	(<17)	-	(<18)
>5,000 <50,000	12	3	-	(<17)	-	(<18)
>50,000	38	9	63 ± 13	26	-	(<18)

[a]Negative response is based on a less than 2-fold above background increase in the number of revertants per plate at highest dose tested. "Less than" calculations are based on background values of 16 for TA98 and 180 for TA100, and maximum dose levels of 0.4 mL sample.

Table II. Halogenated Compounds Identified in Chlorinated Humic Acid Solutions by GC/MS Analysis Following Liquid/Liquid Extraction, Closed-Loop Stripping, or Steam Distillation

Compound	Method Identified			Ames Test Mutagenicity
	Solvent Extracts	Closed-Loop Stripping	Steam Distillate	
Trihalomethanes				
•Chloroform[a]	+	+	+	-
Nitriles				
Chloroacetonitrile[a]	-	-	+	-
•Dichloroacetonitrile[a]	+	+	+	+
•Trichloroacetonitrile[a]	+	+	+	-
Dichloropropanenitrile	-	-	+	?
Dichloropropenenitrile	+	+	+	?
•Trichloropropenenitrile	+	+	+	?
Acids				
•Dichloroacetic acid[a]	+	-	-	-
•Trichloroacetic acid[a]	+	-	-	-
Aldehydes				
•Dichloroacetaldehyde	-	-	+	?
•Trichloroacetaldehyde	-	-	+	?
Dichloropropanal	-	-	+	+
Trichloropropanal	-	-	+	+
2-Chloropropenal[a]	-	-	+	+
2,3-Dichloropropenal	-	-	+	+
3,3-Dichloropropenal[a]	+	+	+	+
2,3,3-Trichloropropenal[a]	-	-	+	+
Trichlorobutanal	-	-	+	?
Dichlorobutenal	-	-	+	?
Ketones				
Chloropropanone	+	-	+	-
•1,1-Dichloropropanone[a]	+	+	+	+
1,3-Dichloropropanone[a]	+	-	+	+
•1,1,1-Trichloropropanone[a]	+	+	+	+
1,1,3-Trichloropropanone[a]	+	-	+	+
1,1,1,3-Tetrachloropropanone[a]	+	-	+	?
1,1,3,3-Tetrachloropropanone[a]	+	-	+	+
Pentachloropropanone[a]	+	-	-	+
3-Chloro-2-butanone	-	-	+	?
•1,1-Dichloro-2-butanone	-	+	+	?

Compound	Method Identified: Solvent Extracts	Closed-Loop Stripping	Steam Distillate	Ames Test Mutagenicity
1,3-Dichloro-2-butanone	-	-	+	?
•3,3-Dichloro-2-butanone	+	+	-	?
•1,1,1-Trichloro-2-butanone	-	+	+	?
1,1,3-Trichloro-2-butanone	-	-	+	?
1-Chloro-3-buten-2-one	-	-	+	?
3-Chloro-3-buten-2-one	-	-	+	?
Trichlorocyclopentenedione	+	-	+	?
Tetrachloro-3-buten-2-one	+	-	+	?
Pentachloro-3-buten-2-one	-	+	+	?
Miscellaneous				
•Hexachloroethane[a]	-	+	-	-
•Pentachloropropene	+	+	-	+
Tetrachlorocyclopropene	-	+	-	?
•Hexachlorocyclopentadiene[a]	+	+	-	-
Tetrachlorothiophene	-	+	-	?
2,4,6-Trichlorophenol	+	-	-	-

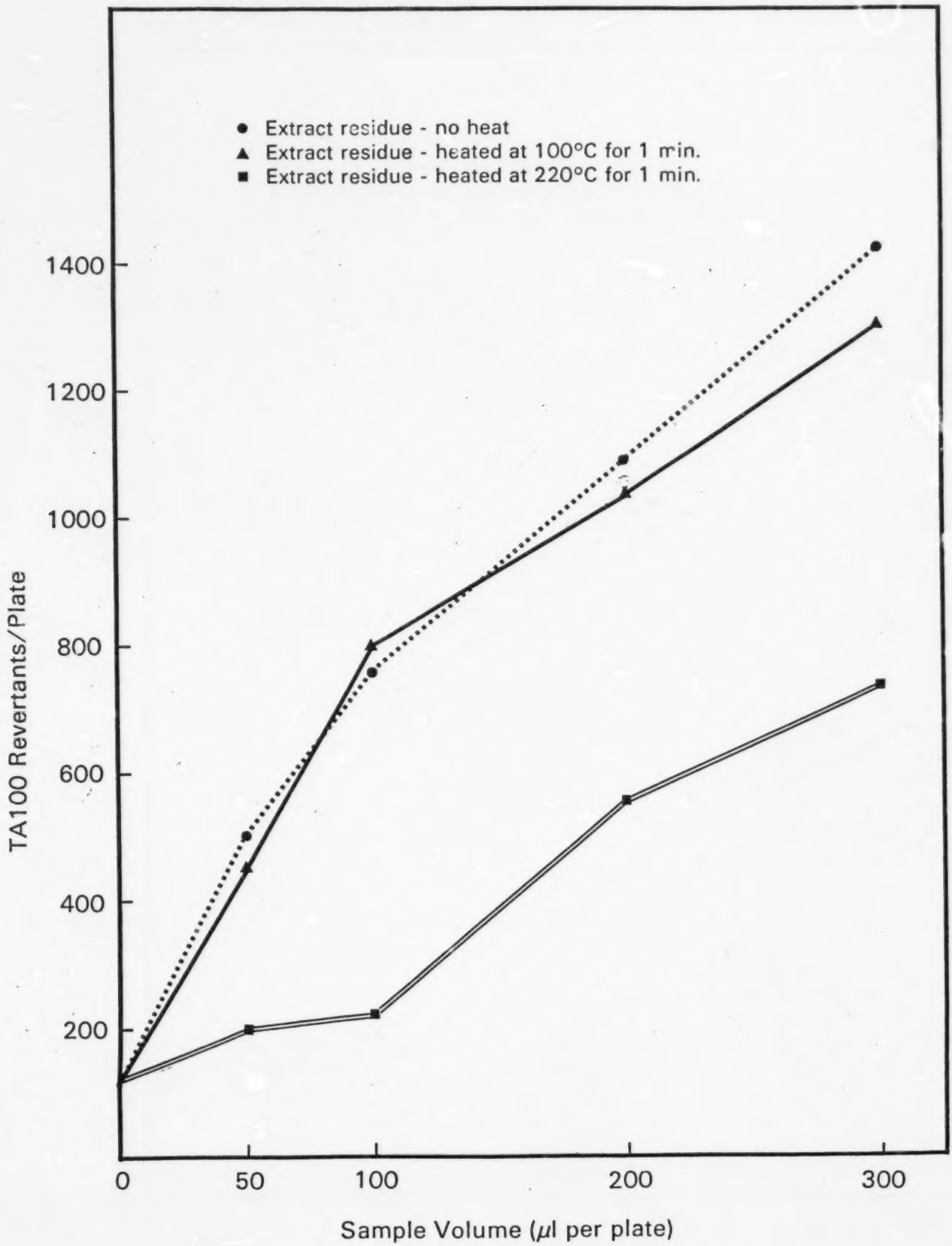
[a]Compounds for which quantifications were made by Coleman et al.[9]
•Compounds identified in drinking water

Table III. Fractionation of Mutagenic Activity in Chlorinated Humic Material By Steam Distillation

Sample	Fraction	TA100 Mutagenicity	
		Net revertants per ml[a]	% Recovery
Non-Chlorinated	Original	N.D.	---
Non-chlorinated	Pot	N.D.	---
Non-Chlorinated	Distillate	N.D.	---
Chlorinated	Original	2,380	(100)
Chlorinated	Pot	149	6.3
Chlorinated	Distillate	318	13.4
Chlorinated	Pot & Distillate Combined	443	18.6
Chlorinated	Original-- Refluxed for 2 Hours	1,140	47.9

[a]Results represent combined data from three experiments. Assays were conducted using 4 dose levels with triplicate plates per dose. Mutagenicity values are calculated from initial slopes.
N.D. = not detected.

Figure 1. Effect of temperature on the mutagenic activity of chlorinated humic acid extracts.



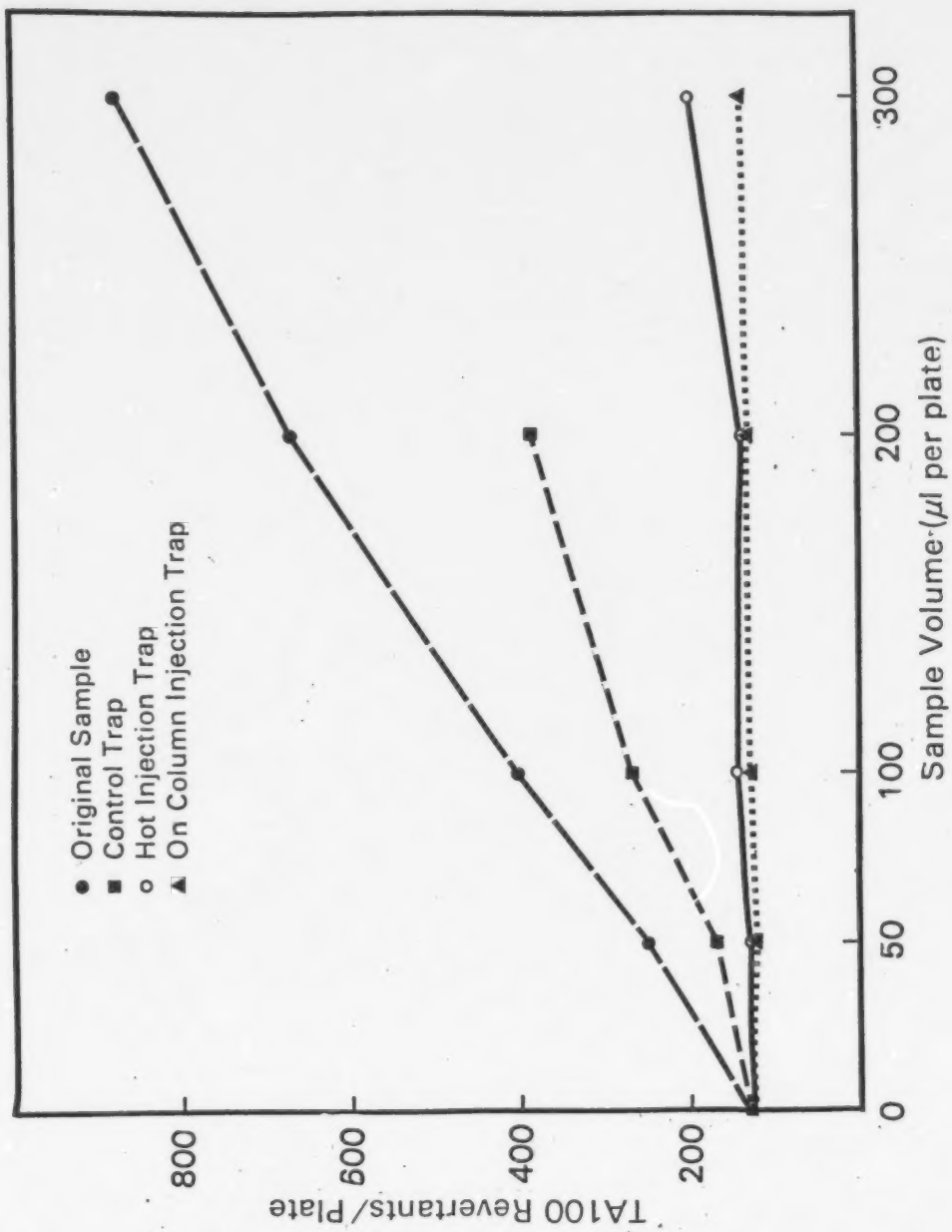


Figure 2. Comparison of GC injection methods for recovery of mutagenic activity from chlorinated humic acid extracts.